

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Katherine GALVIN *et al.*

Application No.: To Be Assigned

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For: COMPOSITIONS AND METHODS  
FOR TREATMENT AND  
DIAGNOSIS OF  
CARDIOVASCULAR DISEASE

Attorney Docket No.: 7853-233-999

**PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115**

Assistant Commissioner for Patents  
**BOX PATENT APPLICATION**  
Washington, D.C. 20231

Sir:

Prior to examination on the merits, please enter the following amendments and remarks. Applicant submits herewith: (a) Exhibit A, a marked version of the amended Specification; (b) Exhibit B, a marked version of the amended Claims; and (c) Exhibit C, a copy of the pending claims.

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to PENNIE & EDMONDS LLP Deposit Account No. 16-1150.

## THE AMENDMENTS

### IN THE SPECIFICATION:

A marked up version of the following amended paragraph is attached hereto as Exhibit A. Matter that has been deleted from the paragraph is indicated by brackets and matter that has been added to the paragraph is indicated by underlining.

Please amend the specification as follows:

Please replace the paragraphs beginning at Page 1, line 1 through line 10:

#### COMPOSITIONS AND METHODS FOR THE TREATMENT AND DIAGNOSIS OF CARDIOVASCULAR DISEASE

#### Cross-Reference to Related Applications

This application is a continuation of Application No. 09/288,292, filed April 8, 1999, which is a continuation-in-part of Application Serial No. 08/870,434, filed June 6, 1997, which is a continuation-in-part of co-pending Application Serial No. 08/799,910, filed February 13, 1997, which claims priority benefit under 35 U.S.C. §119(e) of provisional Application Serial No. 60/011,787, filed February 16, 1996; and this application is a continuation-in-part of Application Serial No. 08/485,573, filed June 7, 1995, which is a continuation-in-part of co-pending Application Serial No. 08/386,844, filed February 10, 1995, each of which is incorporated herein by reference in its entirety.

Please replace the paragraphs beginning at page 11, line 15 through line 25 with the following:

#### 4. DESCRIPTION OF THE FIGURES

FIGS. 1A-1D. Nucleotide sequence (SEQ ID NO:1) and encoded amino acid sequence (SEQ ID NO:2) of the fchd531 gene.

FIGS. 2A-2E. Nucleotide sequence (SEQ ID NO:3) and encoded amino acid sequence (SEQ ID NO:4) of the fchd540 gene.

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FIGS. 3A-3C. Nucleotide sequence (SEQ ID NO:5) and encoded amino acid sequence (SEQ ID NO:6) of the fchd545 gene.

FIGS. 4A-4B. Nucleotide sequence (SEQ ID NO:7) and encoded amino acid sequence (SEQ ID NO:8) from the fchd602 gene.

FIGS 5A-5B. Nucleotide sequence (SEQ ID NO:9) and encoded amino acid sequence (SEQ ID NO:10) from the fchd605 gene.

FIGS. 6A-6D. Nucleotide sequence (SEQ ID NO:11) and encoded amino acid sequence (SEQ ID NO:12) of the rchd534 gene.

Please replace the paragraphs beginning at Page 12, line 13 through line 14:

FIGS. 10A-B. DNA (SEQ ID NO:45) and encoded amino acid sequence (SEQ ID NO:46) of the rchd534-long cDNA encoding the rchd534-long protein.

Please replace the paragraph beginning at page 26, line 5 through line 10 with the following:

#### 5.4.1 DIFFERENTIALLY EXPRESSED AND PATHWAY GENE SEQUENCES

The differentially expressed and pathway genes of the invention are listed below, in Table 1. Differentially expressed and pathway gene nucleotide sequences are shown in FIGS. 1A-1D, 2A-2E, 3A-3C, 4A-4B, 5A-5B, 6A-6D and 8.

Please replace the paragraph bridging page 26 and 27 beginning at page 26, line 33 through page 27, line 3 with the following:

The genes listed in Table 1 may be obtained using cloning methods well known to those skilled in the art, including but not limited to the use of appropriate probes to detect the genes within an appropriate cDNA or gDNA (genomic DNA) library. (See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, which is incorporated by reference herein in its entirety). Probes for the novel sequences reported herein may be obtained directly from the isolated clones deposited with

the ATCC, as indicated in Table 2, below. Alternatively, oligonucleotide probes for the novel genes may be synthesized based on the DNA sequences disclosed herein in FIGS 1A-5B.

Please replace page 28 with the attached revised page.

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# Differentially Expressed and Pathway Genes

Gene	Seq. ID #	Paradigm of Original Detection	Paradigm Expr. Pattern	Cell Type Detected in	Ref	Seq.
feh531	1	D (Section 5.1.1.6)	↓	Endothelial	New,1	FIGS 1A-1A-1D
feh540	3	D	↑	Endothelial	New,2	FIGS 2A-2E
feh545	5	D	↓	Endothelial	New,3	FIGS. 3A-3C
feh602	7	A (Section 5.1.1.1)	↑	Monocytes	New,4	FIG 4A-4B
feh605	9	A	↑	Monocytes	New,5	FIGS 5A-5B
reh534	11	D	↑	Endothelial		FIGS. 6A-6B

- 1 GenBank accession number U05343.
- 2 Drosophila *Mothers against dpp* (*Mad*), Sekelsky et al , 1995, Genetics 139, 1347-1358.
- 3 Human Voltage-dependent Anion Channel, Blachly-Dyson, E., et al , 1993, J. Biol. Chem. 268, 1835-1841; and EST T24012
- 4 Rat Cl-6, Diamond, R.H., et al., 1993, J. Biol. Chem. 268: 15185-15192
- 5 Mouse gly96, Charles, C.H., et al., 1993, Oncogene 8: 797-801; and EST T49532.

Please replace the paragraph bridging pages 29 and 30 beginning at page 29, line 14 through page 30, line 2 with the following:

As used herein, "differentially expressed gene" (i.e. target and fingerprint gene) or "pathway gene" refers to (a) a gene containing at least one of the DNA sequences disclosed herein (as shown in FIGS. 1A-6D), or contained in the clones listed in Table 2, as deposited with the ATCC; (b) any DNA sequence that encodes the amino acid sequence encoded by the DNA sequences disclosed herein (as shown in FIGS. 1A-6D), contained in the clones, listed in Table 2, as deposited with the ATCC or contained within the coding region of the gene to which the DNA sequences disclosed herein (as shown in FIGS. 1A-6D) or contained in the clones listed in Table 2, as deposited with the ATCC, belong; (c) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, contained in the clones listed in Table 2, as deposited with the ATCC, or contained within the coding region of the gene to which the DNA sequences disclosed herein (as shown in FIGS. 1A-6D) or contained in the clones listed in Table 2, as deposited with the ATCC, belong, under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a gene product functionally equivalent to a gene product encoded by sequences contained within the clones listed in Table 2; and/or (d) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, (as shown in FIGS. 1A-6D) contained in the clones listed in Table 2, as deposited with the ATCC or contained within the coding region of the gene to which DNA sequences disclosed herein (as shown in FIGS. 1A-5B) or contained in the clones, listed in Table 2, as deposited with the ATCC, belong, under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet which still encodes a functionally equivalent gene product.

Please replace the paragraph bridging pages 32 and 33 beginning at page 32, line 30 through page 33, line 5 with the following:

5.4.2. DIFFERENTIALLY EXPRESSED AND PATHWAY GENE PRODUCTS

Differentially expressed and pathway gene products include those proteins encoded by the differentially expressed and pathway gene sequences described in Section 5.4.1, above. Specifically, differentially expressed and pathway gene products may include differentially expressed and pathway gene polypeptides encoded by the differentially expressed and pathway gene sequences contained in the clones listed in Table 2, above, as deposited with the ATCC, or contained in the coding regions of the genes to which DNA sequences disclosed herein (in FIGS. 1A-6D) or contained in the clones, listed in Table 2, as deposited with the ATCC, belong, for example.

Please replace the paragraphs beginning at Page 82, line 13 through line 33:

PCR reactions: 13 µl of reaction mix was added to each tube of a 96 well plate on ice. The reaction mix contained 6.4 µl H<sub>2</sub>O, 2 µl 10x PCR Buffer (Perkin-Elmer), 2 µl 20 µM dNTP's, 0.4 µl <sup>35</sup>S dATP (12.5 µCi/µl; 50 µCi total) (Dupont/NEN), 2 µl forward (for-) primer (10 µM) (Operon), and 0.2 µl AmpliTaq Polymerase (5 units/µl) (Perkin-Elmer). Next, 2 µl of reverse (rev-) primer (T<sub>11</sub>XX, 10 µM) were added to the side of each tube followed by 5 µl of cDNA also to the sides of the tubes, which were still on ice. The specific primers used in each experiment were as follows:

fchd602: rev-T<sub>11</sub>XC (SEQ ID NO:13) and for-GTGAGGCGTC (SEQ ID NO:14)

fchd605: rev-T<sub>11</sub>XC (SEQ ID NO:13) and for-TGGACCGGTG (SEQ ID NO:15)

Tubes were capped and mixed, and brought up to 1000 RPM in a centrifuge then returned immediately to ice. The PCR machine (Perkin-Elmer 9600) was programmed for differential display as follows:

94°C 2 min.

\*94°C 15 sec.

\*40°C 2 min.

\* = X40

\*ramp 72°C 1 min.

\*72°C 30 sec.  
72°C 5 min.  
4°C hold

Please replace the paragraph bridging pages 87 and 88 beginning at page 87, line 23 through page 88, line 9 with the following:

The fchd602 gene produced a 2.5kb mRNA that was up-regulated after 5 hours of treatment with oxidized LDL, minimally oxidized LDL, and lysophosphatidylcholine. No message was detected in untreated or native LDL treated control monocytes. The amplified DNA sequence was used to recover a cDNA of approximately 875 bp comprising an open reading frame encoding approximately 182 amino acids. The DNA sequence and encoded amino acid sequence of this cDNA from the fchd602 gene is shown in FIGS. 4A-4B. The open reading frame has 88% sequence similarity to the rat Cl-6 gene, which is induced in regenerating rat liver, is insulin inducible, and also contains multiple transmembrane domains (Diamond, R.H., et al., 1993, J. Biol. Chem. 268: 15185-15192).

The fchd605 gene produced a 1.5kb mRNA that is up-regulated after 5 hours treatment with oxidized LDL, and to a lesser degree with native LDL, as compared to untreated monocytes. The amplified DNA was sequenced and used to recover a cDNA of approximately 2.2kb, which was sequenced to reveal a partial open reading frame of approximately 258 bp, encoding approximately 86 amino acids. The DNA sequence and encoded amino acid sequence from the fchd605 gene is shown in FIGS. 5A-5B. The sequence has similarity to the mouse gly96 gene, which encodes a cytokine inducible glycosylated protein expressed in mouse lung, testes, and uterus.

Page 89, line 20 through line 29:

For induction, second passage HUVEC's were plated on tissue culture-treated polystyrene and subjected to 10 dyn/cm<sup>2</sup> laminar flow for 1 and 6 hr. as described (1994, J. Clin. Invest. 94: 885-891) or 3-10 dyn/cm<sup>2</sup> turbulent flow as previously described (1986, Proc. Natl. Acad. Sci. U.S.A. 83: 2114-2117). RNA was isolated as described, above, in Section 6.1. Differential display, Northern analysis, RT-PCR, subcloning, and DNA sequencing were carried out as described, above, in Section 6.1.2. Specific primers used in differential display were as follows:



fchd531: for-T<sub>11</sub>XA (SEQ ID NO:16) and rev-AGACGTCCAC (SEQ ID NO:17)  
 fchd540: for-T<sub>11</sub>XA (SEQ ID NO:16) and rev-ACTTCGCCAC (SEQ ID NO:18)  
 fchd545: for-T<sub>11</sub>XC (SEQ ID NO:13) and rev-TCGGACGTGA (SEQ ID NO:19)

Please replace the paragraphs bridging pages 90 and 91 beginning at page 90, line 15 through page 91, line 8 with the following:

## 7.2 RESULTS

An amplified fchd531 fragment obtained from differential display was subcloned and sequenced, and used to obtain a 1.9 kb cDNA containing the entire fchd531 coding region. The DNA sequence and encoded amino acid sequence of the novel fchd531 gene is shown in FIGS. 1A-1D. The fchd531 gene encodes a 570 amino acid polypeptide, and has 94% sequence similarity to the mouse penta zinc finger gene (Pzf) (GenBank accession number U05343). Northern analysis of HUVEC's which were subjected turbulent and laminar shear stress demonstrated that the fchd531 gene produces an approximately 5 kb message which is down-regulated under laminar shear stress, but not turbulent shear stress, compared with the static control.

The fchd540 gene was detected as an up-regulated message under shear stress. The amplified fragment was used to probe a Northern blot containing samples from HUVECs treated with laminar shear stress. A 4.4 kb fchd540 mRNA is up-regulated after 6 hours treatment with laminar shear stress. The fchd540 gene is not induced by IL-1 by the method of Paradigm C, (Section 5.1.1.5, above). The amplified fragment was sequenced and used to obtain a 2.7 kb cDNA containing the entire fchd540 coding region. The DNA sequence and encoded amino acid sequence from the fchd540 gene is shown in FIGS. 2A-2E. The fchd540 gene encodes a 426 amino acid polypeptide and has sequence similarity to the Drosophila Mad gene (Sekelsky et al., 1995, Genetics 139: 1347-1358).

The fchd545 gene was detected as a down-regulated message under shear stress. Northern analysis revealed that the fchd545 gene produces a 1.4kb message which is down regulated by turbulent shear stress, but not by laminar shear stress, as compared with static control. The amplified fragment was sequenced and used to isolate a 1.4kb cDNA containing the complete fchd545 coding sequence. The DNA sequence and encoded amino acid

sequence of the fchd545 gene is shown in FIGS. 3A-3C. The fchd545 gene encodes a 283 amino acid polypeptide which has 73% sequence similarity to the human Voltage-dependent Anion Channel (Blachly-Dyson, E., et al., 1993, J. Biol. Chem. 268: 1835-1841). Northern analysis of a commercially available (Clontech, Palo Alto, California) northern blot revealed that the fchd545 gene is expressed in human heart, smooth muscle, and testes.

Please replace the paragraph bridging pages 93 and 94 beginning at page 93, line 12 through page 94, line 4 with the following:

The peptides used are summarized below:

fchd545 Peptide Antigens

Name	Position	Sequence
fchd545.1	48-63	YTDTGKASGNLETKYK (SEQ ID NO:43)
fchd545.2	107-121	TGKKSGKLKASYKRD (SEQ ID NO:44)

11. EXAMPLE: THE RCHD534 AND FCHD540 GENE PRODUCTS INTERACT

The novel fchd540 gene and its nucleotide sequence is described in Section 7, above. The fchd540 gene shares homology with the *Drosophila* Mad gene. The rchd534 gene (described in Applicant's co-pending Application No. 08/485,573, filed June 7, 1995, which is incorporated by reference in its entirety herein) is another gene that is up-regulated in endothelial cells by shear stress. Subsequent to Applicant's discovery of the rchd534 gene, the rchd534 gene has been referred to in published reports as Smad6. As used herein, rchd534 and Smad6 may be used interchangeably. The DNA and encoded amino acid sequence of the rchd534 gene is shown in FIGS. 6A-6D. The rchd534 gene was deposited in the Agricultural Research Service Culture Collection (NRRL) in microorganism FCHD534 on June 6, 1995 and assigned the NRRL Accession No. B-21459. The rchd534 gene also shares homology with the *Drosophila* Mad gene. Mad genes have been shown to play a role in the TGF- $\beta$  signalling pathway (Sekelsky et al., 1995, Genetics 139: 1347-1358; Chen et al., 1996, Nature 383: 691-696; Serra, et al., 1996, Nature Medicine 2: 390-391). TGF- $\beta$  signalling is considered to be beneficial to atherosclerosis and restenosis (Border et al., 1995,

Nature Medicine 1: 1000; Grainger, et al., 1995, Nature Medicine 1: 1067-1073; Kojima, et al., 1991, J. Cell Biol. 113: 1439-1445; Nikol, et al., 1992, J. Clin. Invest. 90: 1582-1592).

Please replace the paragraph beginning at page 101, line 18 through line 27 with the following:

#### 11.3.5 EFFECT OF EXPRESSION ON TGF- $\beta$ SIGNALLING

The effect of both rchd534 and fchd540 on the TGF- $\beta$  signalling pathway was tested in vitro. Primary BAECs were transfected with a construct called p3TP-Lux, containing a TGF- $\beta$  responsive promoter fused to a reporter gene (Wrana et al., 1994, Nature 370: 341-347). The rchd534 gene or the fchd540 gene in pCI expression vectors (Promega) was transfected with and without MADR1 (pCMV5MADR1-Flag, Hoodless et al. 1996 Cell 85: 489-500) or MADR2 (pCMV5MADR2-Flag, Eppert et al. 1996 Cell 86: 543-552). The TGF- $\beta$  response was induced 20-fold by either MADR1 or MADR2. Co-expression of either rchd534 or fchd540 completely eliminated this induction. Thus, the rchd534 and fchd540 proteins inhibited MADR1- and MADR2-mediated TGF- $\beta$  signalling in endothelial cells. To confirm the specificity of this inhibitory effect, site specific mutants of both rchd534 or fchd540 were constructed, based on known mutations identified in Drosophila homologues, that would be predicted to disrupt MAD-like signaling functions (Sekelsky et al., 1995, Genetics 139:1347-58; Raftery, 1995, Genetics 139:241-54; Newfeld et al., 1996, Development 122:2099-108; Wiersdorff et al., 1996, Development 122:2153-62). Unlike wild type rchd534 and fchd540, these mutant proteins were unable to inhibit the activation of the p3TP promoter in response to TGF- $\beta$ . The expression levels of the mutant and wild-type proteins were comparable indicating the loss of function was not due to secondary instability.

Please replace the paragraphs bridging pages 102 and 103 beginning at page 102, line 14 through page 105, line 3 with the following:

#### Antisense:

- a) 5'-CATTTTCATTTTCATACAA-3' (SEQ ID NO:20) which is complementary to nucleotides -14 to +3 of rchd534 in FIG 6A.

- b) 5'-CATTTTCATTTTCATACAATATATG-3' (SEQ ID NO:21) which is complementary to nucleotides -20 to +3 of rchd534 in FIG 6A.
- c) 5'-CATTTTCATTTTCATACAATATATGGCCTTT-3' (SEQ ID NO:22) which is complementary to nucleotides -26 to +3 of rchd534 in FIG 6A.
- d) 5'-CATTTTCATTTTCATACAATATATGGCCTTTTGTGGC-3' (SEQ ID NO:23) which is complementary to nucleotides -32 to +3 of rchd534 in FIG 6A.
- e) 5'-GGACATTTTCATTTTCATACAATATATGGCCTTTTGT-3' (SEQ ID NO:24) which is complementary to nucleotides -29 to +6 of rchd534 in FIG 6A.
- f) 5'-TTCATTTTCATACAATATATGGCCTTTTGT-3' (SEQ ID NO:25) which is complementary to nucleotides -29 to -1 of rchd534 in FIG 6A.
- g) 5'-TCATACAATATATGGCCTTTTGT-3' (SEQ ID NO:26) which is complementary to nucleotides -29 to -7 of rchd534 in FIG 6A.
- h) 5'-AATATATGGCCTTTTGT-3' (SEQ ID NO:27) which is complementary to nucleotides -29 to -13 of rchd534 in FIG 6A.

The following antisense molecules can be used to inhibit the expression of the fchd540 gene:

- a) 5'-CATGCGGGGCGAGGAGG-3' (SEQ ID NO:28) which is complementary to nucleotides -14 to +3 of fchd540 in FIG 2A.
- b) 5'-CATGCGGGGCGAGGAGGCGAGGA-3' (SEQ ID NO:29) which is complementary to nucleotides -20 to +3 of fchd540 in FIG 2A.
- c) 5'-CATGCGGGGCGAGGAGGCGAGGAGAAAAG-3' (SEQ ID NO:30) which is complementary to nucleotides -26 to +3 of fchd540 in FIG 2A.

- d) 5'-CATGCGGGGCGAGGAGGCGAGGAGAAAAGTCGTTT-3' (SEQ ID NO:31) which is complementary to nucleotides -32 to +3 of fchd540 in FIG 2A.
- e) 5'-GAACATGCGGGGCGAGGAGGCGAGGAGAAAAGTCG-3' (SEQ ID NO:32) which is complementary to nucleotides -29 to +6 of fchd540 in FIG 2A.
- f) 5'-GCGGGGCGAGGAGGCGAGGAGAAAAGTCG-3' (SEQ ID NO:33) which is complementary to nucleotides -29 to -1 of fchd540 in FIG 2A.
- g) 5'-CGAGGAGGCGAGGAGAAAAGTCG-3' (SEQ ID NO:34) which is complementary to nucleotides -29 to -7 of fchd540 in FIG 2A.
- h) 5'-GGCGAGGAGAAAAGTCG-3' (SEQ ID NO:35) which is complementary to nucleotides -29 to -13 of fchd540 in FIG 2A.

#### Ribozymes:

The central, catalytic portion of a hammerhead ribozyme molecule consist of the following sequence:

5'-CAAAGCNGNXXXXNCNGAGNAGUC-3' (SEQ ID NO:36);

wherein the 5'-proximal CA bases hybridize to a complementary 5'-UG-3' in the target mRNA. The first four underlined bases form a stem by base pairing with the second set of underlined bases, with the intervening bases, shown as X's, forming a non-pairing loop. In order to hybridize to a target mRNA, a hammerhead ribozyme contains additional bases flanking each end of the central segment shown above. The 5' ribozyme flanking segment is complementary to the respective flanking sequences immediately 3' to the target UG; and the 3' flanking segment is complementary to the respective flanking sequence beginning two bases upstream of the target U, and extending 5'-ward (in effect, skipping the first base upstream of the target U). Cleavage occurs between first and second bases upstream of (*i.e.*, 5' to) the U in the target 5'-UG-3' site.

The following ribozyme molecules can be used to inhibit the expression of the rhd534 gene:

- a) 5'-GGUGGAGCCCCAGGGCAUUACCUCAAAGCNGNXXXXXNCNGAGNAGUC GUGGGCAAGGUGGGCACUCAGGUGGG-3' (SEQ ID NO:37) which will cleave the rhd534 mRNA between nucleotides 716 and 717 in FIG 6A.
- b) 5'-GUGUCUCUAUGGGUUUGCCCAAAGCNGNXXXXXNCNGAGNAGUCUCUG GACAUUUCAUUUCAC-3' (SEQ ID NO:38) which will cleave the rhd534 mRNA between nucleotides 1040 and 1041 in FIG 6B.
- c) 5'-GGCCUCUCGCCGUCGGGCUCCUUGCUGAGCAAAGCNGNXXXXXNCNG AGNAGUCGAUGCCGAAGCCGAUCUUGCUGCGCG-3' (SEQ ID NO:39) which will cleave between nucleotides 1421 and 1422 in FIG 6B.

The following ribozyme molecules can be used to inhibit the expression of the fhd540 gene:

- a) 5'-CGUUUGCCUGCUAAGGAGCGAACAAAGCNGNXXXXXNCNGAGNAGUCG AUGUUUCUUUGUGAGUCGGGCGCCG-3' (SEQ ID NO:40), which will cleave the fhd540 mRNA between nucleotides -53 and -52 in FIG 2A.
- b) 5'-CGCCGGACGAGCGCAGAUUCGUUUGGUCCUGAACAAAGCNGNXXXXXNC NGAGNAGUCCGGGGCGAGGAGGCGAGGAGAAAAGUCG-3' (SEQ ID NO:41), which will cleave the fhd540 mRNA between nucleotides -1 and +1 in FIG 2A.
- c) 5'-GGAGUAAGGAGGGGGGGGAGACUCUAGUUCGCAAAGCNGNXXXXXNCN GAGNAGUCAGUCGGCUAAGGUGAUGGGGGUUGCAGCACACC-3' (SEQ ID NO:42) which will cleave the fhd540 mRNA between nucleotides +602 and +603 in FIG 2B.

Please replace the paragraphs beginning at page 112, line 1 through line 21 with the following:

14.1 IDENTIFICATION AND CHARACTERIZATION OF  
THE RCHD534-LONG SPLICEOFORM AND PROTEIN

A human heart cDNA library (Stratagene, LaJolla, CA) was screened with a probe containing nucleotides 400-700 of the fchd540 (see FIG. 2B) under the following hybridization conditions: hybridization overnight at 65°C, washing with 2xSSC and 0.1% SDS for 20 minutes at room temperature, followed two washes with 0.2xSSC and 0.1%SDS for 20 minutes at 65°C. A positive clone was found to encode a novel protein, related to the rchd534 protein, that was designated the rchd534-long protein. The rchd534-long protein, like the rchd534 protein, has an MH2 domain. In addition, the rchd534-long protein has an MH1 domain not present in rchd534. The original clone isolated from the Stratagene heart library contained incorrect sequence, including two stop codons, between the MH1 and MH2 domain coding regions. Therefore, cDNA prepared from human heart mRNA (Clontech, Palo Alto, CA) was used as template for the PCR reaction to isolate the correct cDNA sequence in the region spanning the MH1 and MH2 domain coding regions. The following two pairs of nested primers were used:

Pair 1: A) 5'-GAGGCTGCGGCCGCTCCGAAGTCC-3' (SEQ ID NO:47)

B) 5'-CTCCGCCGGGGCCGCCACTATCT-3' (SEQ ID NO:48)

Pair 2: A) 5'-CCGGGACGCAGTGGGACAG-3' (SEQ ID NO:49)

B) 5'-CGGGGAGTTGACGAAGATGG-3' (SEQ ID NO:50)

Please replace the paragraph beginning at page 112, line 29 through line 35 with the following:

This correct cDNA encoding the rchd534-long protein was cloned into the TA cloning vector (Invitrogen) to create plasmid pHL6TA1A, which was deposited with the American Type Culture Collection on February 6, 1998 as Accession No. 209615. The cDNA sequence of the rchd534-long spliceoform encoding the entire rchd534-long protein is shown in FIG. 10A-10B. The rchd534-long nucleotide sequence is 93% identical to the nucleotide sequence

of the mouse SMAD6 gene (Imamura et al., 1997, Nature 389: 622-626). The rchd534-long sequence was reported in Hata et al., 1998, Genes and Development 12: 186-197.

### **IN THE CLAIMS**

Please cancel Claims 14-25 and 29-33, without prejudice to the subject matter therein.

Please amend the claims as follows.

1. (Once Amended) A homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, and wherein said animal displays a cardiovascular disease symptom.
2. (Once Amended) The transgenic animal of claim 1, wherein said cardiovascular disease symptom is hyperplasia, thickening of at least one cardiac valve, cardiac outflow tract development defects, cardiovascular calcification, epicardial vascular malformations, endocardial vascular malformation, or defects in the regulation of vascular tone.
3. (Once Amended) The transgenic animal of claim 1, wherein said cardiovascular disease symptom is cardiovascular calcification.
4. (Once Amended) The transgenic animal of claim 1, wherein said cardiovascular disease symptom is aortic or valvular calcification.
6. (Once Amended) A cell having a mutated rchd534 gene isolated from the transgenic animal of claim 1, wherein said cell is isolated from tissue displaying a cardiovascular disease symptom.
10. (Once Amended) A method of producing a homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, comprising introducing a



polynucleotide into an embryonic cell of said animal through homologous recombination with an endogenous rchd534 gene, and wherein said transgenic animal displays a cardiovascular disease symptom.

26. (Once Amended) A method for identifying a substance for treating or preventing cardiovascular disease, comprising administering said substance to a homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, wherein said transgenic animal displays a cardiovascular disease symptom, and wherein amelioration of said cardiovascular disease symptom indicates a substance effective in the treatment or prevention of cardiovascular disease.

### REMARKS


The references to Figures 1A-1B (now 1A-1D), 2A-2B (now 2A-2E), 3 (now 3A-3C), 4 (now 4A-4B), 5 (now 5A-5B), and 6A-6B (now 6A-6D), in the specification have been amended to conform these references to the designations of these figures contained in the formal drawings, in compliance with the requirements of 37 C.F.R. § 1.84. Accordingly, the amendments merely conform the specification to designations for the figures in the formal drawings and add no new matter.

Claims 1-13 and 26-28 are pending in the above-identified application. Claims 1-33 were pending in this application. Claims 14-25 and 29-33 have been canceled without prejudice to pursue these claims in this or any other continuing application. The amendments to the claims does not constitute new matter as defined in 35 U.S.C. § 132. A marked version of the claims which have been amended, with the amendments indicated by bracketing for deleting and underlining for additions, is attached hereto as Exhibit B. A copy of the pending claims is attached hereto as Exhibit C.

Applicants respectfully request entry of the above-made amendments and the foregoing remarks into the file of the instant application.

Respectfully submitted,

Date February 8, 2002



39,445

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Enclosure

**EXHIBIT A**  
**MARKED VERSION OF THE SPECIFICATION**  
**CONTINUATION APPLICATION OF U.S. APPLICATION NO. 09/288,292**  
**FILED ON FEBRUARY 8, 2002**



**Specification**

Page 1, line 1 through line 10

**COMPOSITIONS AND METHODS FOR THE TREATMENT  
AND DIAGNOSIS OF CARDIOVASCULAR DISEASE**

**Cross-Reference to Related Applications**

This application is a continuation of Application No. 09/288,292, filed April 8, 1999, which is a continuation-in-part of Application Serial No. 08/870,434, filed June 6, 1997, which is a continuation-in-part of co-pending Application Serial No. 08/799,910, filed February 13, 1997, which claims priority benefit under 35 U.S.C. §119(e) of provisional Application Serial No. 60/011,787, filed February 16, 1996; and this application is a continuation-in-part of Application Serial No. 08/485,573, filed June 7, 1995, which is a continuation-in-part of co-pending Application Serial No. 08/386,844, filed February 10, 1995, each of which is incorporated herein by reference in its entirety.

Page 11, line 15 through line 25:

4. **DESCRIPTION OF THE FIGURES**

FIGS. [1A-B] 1A-1D. Nucleotide sequence (SEQ ID NO:1) and encoded amino acid sequence (SEQ ID NO:2) of the fchd531 gene.

FIGS. [2A-B] 2A-2E. Nucleotide sequence (SEQ ID NO:3) and encoded amino acid sequence (SEQ ID NO:4) of the fchd540 gene.

[FIG. 3] FIGS. 3A-3C. Nucleotide sequence (SEQ ID NO:5) and encoded amino acid sequence (SEQ ID NO:6) of the fchd545 gene.

[FIG. 4] FIGS. 4A-4B. Nucleotide sequence (SEQ ID NO:7) and encoded amino acid sequence (SEQ ID NO:8) from the fchd602 gene.

[FIG. 5] FIGS 5A-5B. Nucleotide sequence (SEQ ID NO:9) and encoded amino acid sequence (SEQ ID NO:10) from the fchd605 gene.

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FIGS. 10A-B. DNA (SEQ ID NO:45) and encoded amino acid sequence (SEQ ID NO:46) of the rchd534-long cDNA encoding the rchd534-long protein.

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5.4.1 DIFFERENTIALLY EXPRESSED AND PATHWAY GENE  
SEQUENCES

The differentially expressed and pathway genes of the invention are listed below, in Table 1. Differentially expressed and pathway gene nucleotide sequences are shown in FIGS. 1A-1D, 2A-2E, 3A-3C, 4A-4B, 5A-5B, 6A-6D and 8, 12, 15, 18, 22, 28, 31, and 35].

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The genes listed in Table 1 may be obtained using cloning methods well known to those skilled in the art, including but not limited to the use of appropriate probes to detect the genes within an appropriate cDNA or gDNA (genomic DNA) library. (See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, which is incorporated by reference herein in its entirety). Probes for the novel sequences reported herein may be obtained directly from the isolated clones deposited with the ATCC, as indicated in Table 2, below. Alternatively, oligonucleotide probes for the novel genes may be synthesized based on the DNA sequences disclosed herein in FIGS [1-5] 1A-5B.

Please replace page 28 with the attached revised page.

# Differentially Expressed and Pathway Genes

Gene	Seq. ID #	Paradigm of Original Detection	Paradigm Expr. Pattern	Cell Type Detected in	Ref	Seq.
fchd531	<u>1</u>	D (Section 5.1.1.6)	↓	Endothelial	New,1	[FIG.1] FIGS 1A-1A-1D
fchd540	<u>3</u>	D	↑	Endothelial	New,2	[FIG.2] FIGS 2A-2E
fchd545	<u>5</u>	D	↓	Endothelial	New,3	[FIG.3] FIGS. 3A-3C
fchd602	<u>7</u>	A (Section 5.1.1.1)	↑	Monocytes	New,4	[FIG.4] FIG 4A-4B
fchd605	<u>9</u>	A	↑	Monocytes	New,5	[FIG.5] FIGS 5A-5B
rchd534	<u>11</u>	D	↑	Endothelial		[FIG. 6] FIGS. 6A-6B

1. GenBank accession number U05343

2. *Drosophila Mothers against dpp (Mad)*, Sekelsky et al., 1995, Genetics 139: 1347-1358.

3. Human Voltage-dependent Anion Channel, Blachly-Dyson, E., et al., 1993, J. Biol. Chem. 268: 1835-1841; and EST T24012

4. Rat Cl-6, Diamond, R.H., et al., 1993, J. Biol. Chem. 268: 15185-15192.

5. Mouse gly96, Charles, C.H., et al., 1993, Oncogene 8: 797-801, and EST T49532.

Page 29, line 14 through page 30, line 2:

As used herein, "differentially expressed gene" (i.e. target and fingerprint gene) or "pathway gene" refers to (a) a gene containing at least one of the DNA sequences disclosed herein (as shown in FIGS. [1-6] 1A-6D), or contained in the clones listed in Table 2, as deposited with the ATCC; (b) any DNA sequence that encodes the amino acid sequence encoded by the DNA sequences disclosed herein (as shown in FIGS.[1-6] 1A-6D), contained in the clones, listed in Table 2, as deposited with the ATCC or contained within the coding region of the gene to which the DNA sequences disclosed herein (as shown in FIGS.[1-6] 1A-6D) or contained in the clones listed in Table 2, as deposited with the ATCC, belong; (c) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, contained in the clones listed in Table 2, as deposited with the ATCC, or contained within the coding region of the gene to which the DNA sequences disclosed herein (as shown in FIGS.[1-6] 1A-6D) or contained in the clones listed in Table 2, as deposited with the ATCC, belong, under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a gene product functionally equivalent to a gene product encoded by sequences contained within the clones listed in Table 2; and/or (d) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, (as shown in FIGS.[1-6] 1A-6D) contained in the clones listed in Table 2, as deposited with the ATCC or contained within the coding region of the gene to which DNA sequences disclosed herein (as shown in FIGS.[1-5] 1A-5B) or contained in the clones, listed in Table 2, as deposited with the ATCC, belong, under less stringent conditions, such as moderately stringent conditions,

e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet which still encodes a functionally equivalent gene product.

Page 32, line 30 through page 33, line 5:

#### 5.4.2. DIFFERENTIALLY EXPRESSED AND PATHWAY GENE PRODUCTS

Differentially expressed and pathway gene products include those proteins encoded by the differentially expressed and pathway gene sequences described in Section 5.4.1, above. Specifically, differentially expressed and pathway gene products may include differentially expressed and pathway gene polypeptides encoded by the differentially expressed and pathway gene sequences contained in the clones listed in Table 2, above, as deposited with the ATCC, or contained in the coding regions of the genes to which DNA sequences disclosed herein (in FIGS. [1-6] 1A-6D) or contained in the clones, listed in Table 2, as deposited with the ATCC, belong, for example.

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PCR reactions: 13 µl of reaction mix was added to each tube of a 96 well plate on ice. The reaction mix contained 6.4 µl H<sub>2</sub>O, 2 µl 10x PCR Buffer (Perkin-Elmer), 2 µl 20 µM dNTP's, 0.4 µl <sup>35</sup>S dATP (12.5 µCi/µl; 50 µCi total) (Dupont/NEN), 2 µl forward (for-) primer (10 µM) (Operon), and 0.2 µl AmpliTaq Polymerase (5 units/µl) (Perkin-Elmer). Next, 2 µl of reverse (rev-) primer (T<sub>11</sub>XX, 10 µM) were added to the side of each tube followed by 5 µl of cDNA also to the sides of the tubes, which were still on ice. The specific primers used in each experiment were as follows:

fchd602: rev-T<sub>11</sub>XC (SEQ ID NO:13) and for-GTGAGGCGTC (SEQ ID NO:14)  
fchd605: rev-T<sub>11</sub>XC (SEQ ID NO:13) and for-TGGACCGGTG (SEQ ID NO:15)

Tubes were capped and mixed, and brought up to 1000 RPM in a centrifuge then returned immediately to ice. The PCR machine (Perkin-Elmer 9600) was programmed for differential display as follows:

94°C 2 min.  
\*94°C 15 sec.  
\*40°C 2 min.  
\* = X40                      \*ramp 72°C 1 min.  
\*72°C 30 sec.  
72°C 5 min.  
4°C hold

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The fchd602 gene produced a 2.5kb mRNA that was up-regulated after 5 hours of treatment with oxidized LDL, minimally oxidized LDL, and lysophosphatidylcholine. No message was detected in untreated or native LDL treated control monocytes. The amplified DNA sequence was used to recover a cDNA of approximately 875 bp comprising an open reading frame encoding approximately 182 amino acids. The DNA sequence and encoded amino acid sequence of this cDNA from the fchd602 gene is shown in [FIG.4] FIGS. 4A-4B. The open reading frame has 88% sequence similarity to the rat Cl-6 gene, which is induced in regenerating rat liver, is insulin inducible, and also contains multiple transmembrane domains (Diamond, R.H., et al., 1993, J. Biol. Chem. 268: 15185-15192).

The fchd605 gene produced a 1.5kb mRNA that is up-regulated after 5 hours treatment with oxidized LDL, and to a lesser degree with native LDL, as compared to untreated monocytes. The amplified DNA was sequenced and used to recover a cDNA of approximately 2.2kb, which was sequenced to reveal a partial open reading frame of approximately 258 bp, encoding approximately 86 amino acids. The DNA sequence and encoded amino acid sequence from the fchd605 gene is shown in [FIG.5] FIGS. 5A-5B. The sequence has similarity to the mouse gly96 gene, which encodes a cytokine inducible glycosylated protein expressed in mouse lung, testes, and uterus.



Page 89, line 20 through line 29:

For induction, second passage HUVEC's were plated on tissue culture-treated polystyrene and subjected to 10 dyn/cm<sup>2</sup> laminar flow for 1 and 6 hr. as described (1994, J. Clin. Invest. 94: 885-891) or 3-10 dyn/cm<sup>2</sup> turbulent flow as previously described (1986, Proc. Natl. Acad. Sci. U.S.A. 83: 2114-2117). RNA was isolated as described, above, in Section 6.1. Differential display, Northern analysis, RT-PCR, subcloning, and DNA sequencing were carried out as described, above, in Section 6.1.2. Specific primers used in differential display were as follows:

fchd531: for-T<sub>11</sub>XA (SEQ ID NO:16) and rev-AGACGTCCAC (SEQ ID NO:17)  
fchd540: for-T<sub>11</sub>XA (SEQ ID NO:16) and rev-ACTTCGCCAC (SEQ ID NO:18)  
fchd545: for-T<sub>11</sub>XC (SEQ ID NO:13) and rev-TCGGACGTGA (SEQ ID NO:19)

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## 7.2 RESULTS

An amplified fchd531 fragment obtained from differential display was subcloned and sequenced, and used to obtain a 1.9 kb cDNA containing the entire fchd531 coding region. The DNA sequence and encoded amino acid sequence of the novel fchd531 gene is shown in [FIG.1] FIGS. 1A-1D. The fchd531 gene encodes a 570 amino acid polypeptide, and has 94% sequence similarity to the mouse penta zinc finger gene (Pzf) (GenBank accession number U05343). Northern analysis of HUVEC's which were subjected turbulent and laminar shear stress demonstrated that the fchd531 gene produces an approximately 5 kb message which is down-regulated under laminar shear stress, but not turbulent shear stress, compared with the static control.

The fchd540 gene was detected as an up-regulated message under shear stress. The amplified fragment was used to probe a Northern blot containing samples from HUVECs treated with laminar shear stress. A 4.4 kb fchd540 mRNA is up-regulated after 6 hours treatment with laminar shear stress. The fchd540 gene is not induced by IL-1 by the method of Paradigm C, (Section 5.1.1.5, above). The amplified fragment was sequenced and used to obtain a 2.7 kb cDNA containing the entire fchd540 coding region. The DNA sequence and

encoded amino acid sequence from the fchd540 gene is shown in [FIG.2] FIGS. 2A-2E. The fchd540 gene encodes a 426 amino acid polypeptide and has sequence similarity to the *Drosophila* Mad gene (Sekelsky et al., 1995, Genetics 139: 1347-1358).

The fchd545 gene was detected as a down-regulated message under shear stress. Northern analysis revealed that the fchd545 gene produces a 1.4kb message which is down regulated by turbulent shear stress, but not by laminar shear stress, as compared with static control. The amplified fragment was sequenced and used to isolate a 1.4kb cDNA containing the complete fchd545 coding sequence. The DNA sequence and encoded amino acid sequence of the fchd545 gene is shown in [FIG.3] FIGS. 3A-3C. The fchd545 gene encodes a 283 amino acid polypeptide which has 73% sequence similarity to the human Voltage-dependent Anion Channel (Blachly-Dyson, E., et al., 1993, J. Biol. Chem. 268: 1835-1841). Northern analysis of a commercially available (Clontech, Palo Alto, California) northern blot revealed that the fchd545 gene is expressed in human heart, smooth muscle, and testes.

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The peptides used are summarized below:

#### fchd545 Peptide Antigens

Name	Position	Sequence
fchd545.1	48-63	YTDTGKASGNLETKYK (SEQ ID NO:43)
fchd545.2	107-121	TGKKSGKCLKASYKRD (SEQ ID NO:44)

#### 11. EXAMPLE: THE RCHD534 AND FCHD540 GENE PRODUCTS INTERACT

The novel fchd540 gene and its nucleotide sequence is described in Section 7, above. The fchd540 gene shares homology with the *Drosophila* Mad gene. The rchd534 gene (described in Applicant's co-pending Application No. 08/485,573, filed June 7, 1995, which is incorporated by reference in its entirety herein) is another gene that is up-regulated in endothelial cells by shear stress. Subsequent to Applicant's discovery of the rchd534 gene, the rchd534 gene has been referred to in published reports as Smad6. As used herein,

rchd534 and Smad6 may be used interchangeably. The DNA and encoded amino acid sequence of the rchd534 gene is shown in [FIG.6] FIGS. 6A-6D. The rchd534 gene was deposited in the Agricultural Research Service Culture Collection (NRRL) in microorganism FCHD534 on June 6, 1995 and assigned the NRRL Accession No. B-21459. The rchd534 gene also shares homology with the *Drosophila* Mad gene. Mad genes have been shown to play a role in the TGF- $\beta$  signalling pathway (Sekelsky et al., 1995, Genetics 139: 1347-1358; Chen et al., 1996, Nature 383: 691-696; Serra, et al., 1996, Nature Medicine 2: 390-391). TGF- $\beta$  signalling is considered to be beneficial to atherosclerosis and restenosis (Border et al., 1995, Nature Medicine 1: 1000; Grainger, et al., 1995, Nature Medicine 1: 1067-1073; Kojima, et al., 1991, J. Cell Biol. 113: 1439-1445; Nikol, et al., 1992, J. Clin. Invest. 90: 1582-1592).

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#### 11.3.5 EFFECT OF EXPRESSION ON TGF-B SIGNALLING

The effect of both rchd534 and fchd540 on the TGF- $\beta$  signalling pathway was tested in vitro. Primary BAECs were transfected with a construct called p3TP-Lux, containing a TGF- $\beta$  responsive promoter fused to a reporter gene (Wrana et al., [1994] 1994, Nature 370: 341-347). The rchd534 gene or the fchd540 gene in pCI expression vectors (Promega) was transfected with and without MADR1 (pCMV5MADR1-Flag, Hoodless et al. 1996 Cell 85: 489-500) or MADR2 (pCMV5MADR2-Flag, Eppert et al. 1996 Cell 86: 543-552). The TGF- $\beta$  response was induced 20-fold by either MADR1 or MADR2. Co-expression of either rchd534 or fchd540 completely eliminated this induction. Thus, the rchd534 and fchd540 proteins inhibited MADR1- and MADR2-mediated TGF- $\beta$  signalling in endothelial cells. To confirm the specificity of this inhibitory effect, site specific mutants of both rchd534 or fchd540 were constructed, based on known mutations identified in *Drosophila* homologues, that would be predicted to disrupt MAD-like signaling functions (Sekelsky et al., 1995, Genetics 139:1347-58; Raftery, 1995, Genetics 139:241-54; Newfeld et al., 1996, Development 122:2099-108; Wiersdorff et al., 1996, Development 122:2153-62). Unlike wild type rchd534 and fchd540, these mutant proteins were unable to inhibit the activation of

the p3TP promoter in response to TGF- $\beta$ . The expression levels of the mutant and wild-type proteins were comparable indicating the loss of function was not due to secondary instability.

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Antisense:

- a) 5'-CATTTCATTTCATACAA-3' (SEQ ID NO:20) which is complementary to nucleotides -14 to +3 of rchd534 in [FIG.6] FIG 6A.
- b) 5'-CATTTCATTTCATACAATATATG-3' (SEQ ID NO:21) which is complementary to nucleotides -20 to +3 of rchd534 in [FIG.6] FIG 6A.
- c) 5'-CATTTCATTTCATACAATATATGGCCTTT-3' (SEQ ID NO:22) which is complementary to nucleotides -26 to +3 of rchd534 in [FIG.6] FIG 6A.
- d) 5'-CATTTCATTTCATACAATATATGGCCTTTTGTGGC-3' (SEQ ID NO:23) which is complementary to nucleotides -32 to +3 of rchd534 in [FIG.6] FIG 6A.
- e) 5'-GGACATTTCATTTCATACAATATATGGCCTTTTGT-3' (SEQ ID NO:24) which is complementary to nucleotides -29 to +6 of rchd534 in [FIG.6] FIG 6A.
- f) 5'-TTCATTTCATACAATATATGGCCTTTTGT-3' (SEQ ID NO:25) which is complementary to nucleotides -29 to -1 of rchd534 in [FIG.6] FIG 6A.
- g) 5'-TCATACAATATATGGCCTTTTGT-3' (SEQ ID NO:26) which is complementary to nucleotides -29 to -7 of rchd534 in [FIG.6] FIG 6A.
- h) 5'-AATATATGGCCTTTTGT-3' (SEQ ID NO:27) which is complementary to nucleotides -29 to -13 of rchd534 in [FIG.6] FIG 6A.

The following antisense molecules can be used to inhibit the expression of the fchd540 gene:

- a) 5'-CATGCGGGGCGAGGAGG-3' (SEQ ID NO:28) which is complementary to nucleotides -14 to +3 of fchd540 in [FIG.2] FIG 2A.

- b) 5'-CATGCGGGGCGAGGAGGCGAGGA-3' (SEQ ID NO:29) which is complementary to nucleotides -20 to +3 of fchd540 in [FIG.2] FIG 2A.
- c) 5'-CATGCGGGGCGAGGAGGCGAGGAGAAAAG-3' (SEQ ID NO:30) which is complementary to nucleotides -26 to +3 of fchd540 in [FIG.2] FIG 2A.
- d) 5'-CATGCGGGGCGAGGAGGCGAGGAGAAAAGTCGTTT-3' (SEQ ID NO:31) which is complementary to nucleotides -32 to +3 of fchd540 in [FIG.2] FIG 2A.
- e) 5'-GAACATGCGGGGCGAGGAGGCGAGGAGAAAAGTCG-3' (SEQ ID NO:32) which is complementary to nucleotides -29 to +6 of fchd540 in [FIG.2] FIG 2A.
- f) 5'-GCGGGGCGAGGAGGCGAGGAGAAAAGTCG-3' (SEQ ID NO:33) which is complementary to nucleotides -29 to -1 of fchd540 in [FIG.2] FIG 2A.
- g) 5'-CGAGGAGGCGAGGAGAAAAGTCG-3' (SEQ ID NO:34) which is complementary to nucleotides -29 to -7 of fchd540 in [FIG.2] FIG 2A.
- h) 5'-GGCGAGGAGAAAAGTCG-3' (SEQ ID NO:35) which is complementary to nucleotides -29 to -13 of fchd540 in [FIG.2] FIG 2A.

#### Ribozymes:

The central, catalytic portion of a hammerhead ribozyme molecule consist of the following sequence:

5'-CAAAGCNGNXXXXNCNGAGNAGUC-3' (SEQ ID NO:36);

wherein the 5'-proximal CA bases hybridize to a complementary 5'-UG-3' in the target mRNA. The first four underlined bases form a stem by base pairing with the second set of underlined bases, with the intervening bases, shown as X's, forming a non-pairing loop. In order to hybridize to a target mRNA, a hammerhead ribozyme contains additional bases flanking each end of the central segment shown above. The 5' ribozyme flanking segment is complementary to the respective flanking sequences immediately 3' to the target UG; and the

3' flanking segment is complementary to the respective flanking sequence beginning two bases upstream of the target U, and extending 5'-ward (in effect, skipping the first base upstream of the target U). Cleavage occurs between first and second bases upstream of (*i.e.*, 5' to) the U in the target 5'-UG-3' site.

The following ribozyme molecules can be used to inhibit the expression of the rchd534 gene:

- a) 5'-GGUGGAGCCCCAGGGCAUUACCUCAAAGCNGNXXXXNCNGAGNAGUC GUGGGCAAGGUGGGCACUCAGGUGGG-3' (SEQ ID NO:37) which will cleave the rchd534 mRNA between nucleotides 716 and 717 in [FIG.6] FIG 6A.
- b) 5'-GUGUCUCUAUGGGUUUGCCCCAAAGCNGNXXXXNCNGAGNAGUCUCUG GACAUUUCAUUUCAUAC-3' (SEQ ID NO:38) which will cleave the rchd534 mRNA between nucleotides 1040 and 1041 in [FIG.6] FIG 6B.
- c) 5'-GGCCCUCUCGCCGUCGGGCUCCUUGCUGAGCAAAGCNGNXXXXNCNG AGNAGUCGAUGCCGAAGCCGAUCUUGCUGCGCG-3' (SEQ ID NO:39) which will cleave between nucleotides 1421 and 1422 in [FIG.6] FIG 6B.

The following ribozyme molecules can be used to inhibit the expression of the fchd540 gene:

- a) 5'-CGUUUGCCUGCUAAGGAGCGAACAAAGCNGNXXXXNCNGAGNAGUCG AUGUUUCUUUGUGAGUCGGGCGCCG-3' (SEQ ID NO:40), which will cleave the fchd540 mRNA between nucleotides -53 and -52 in [FIG.2] FIG 2A.
- b) 5'-CGCCGGACGAGCGCAGAU CGUUUGGUCCUGAACAAAGCNGNXXXXNC NGAGNAGUCCGGGGCGAGGAGGCGAGGAGAAAAGUCG-3' (SEQ ID NO:41), which will cleave the fchd540 mRNA between nucleotides -1 and +1 in [FIG.2] FIG 2A.
- c) 5'-GGAGUAAGGAGGGGGGGGAGACUCUAGUUCGCAAAGCNGNXXXXNCN GAGNAGUCAGUCGGCUAAGGUGAUGGGGGUUGCAGCACACC-3' (SEQ ID NO:42) which will cleave the fchd540 mRNA between nucleotides +602 and +603 in [FIG.2] FIG 2B.

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14.1 IDENTIFICATION AND CHARACTERIZATION OF  
THE RCHD534-LONG SPLICEFORM AND PROTEIN

A human heart cDNA library (Stratagene, LaJolla, CA) was screened with a probe containing nucleotides 400-700 of the fchd540 (see [FIG.10] FIG. 2B) under the following hybridization conditions: hybridization overnight at 65°C, washing with 2xSSC and 0.1% SDS for 20 minutes at room temperature, followed two washes with 0.2xSSC and 0.1%SDS for 20 minutes at 65°C. A positive clone was found to encode a novel protein, related to the rchd534 protein, that was designated the rchd534-long protein. The rchd534-long protein, like the rchd534 protein, has an MH2 domain. In addition, the rchd534-long protein has an MH1 domain not present in rchd534. The original clone isolated from the Stratagene heart library contained incorrect sequence, including two stop codons, between the MH1 and MH2 domain coding regions. Therefore, cDNA prepared from human heart mRNA (Clontech, Palo Alto, CA) was used as template for the PCR reaction to isolate the correct cDNA sequence in the region spanning the MH1 and MH2 domain coding regions. The following two pairs of nested primers were used:

Pair 1: A) 5-'GAGGCTGCGGCCGCTCCGAAGTCC-3' (SEQ ID NO:47)

B) 5'-CTCCGCCGGGGCCGCCACTATCT-3' (SEQ ID NO:48)

Pair 2: A) 5'-CCGGGACGCAGTGGGACAG-3' (SEQ ID NO:49)

B) 5'-CGGGGAGTTGACGAAGATGG-3' (SEQ ID NO:50)

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This correct cDNA encoding the rchd534-long protein was cloned into the TA cloning vector (Invitrogen) to create plasmid pHL6TA1A, which was deposited with the American Type Culture Collection on February 6, 1998 as Accession No. 209615. The cDNA sequence of the rchd534-long spliceoform encoding the entire rchd534-long protein is shown in FIG. [12] 10A-10B. The rchd534-long nucleotide sequence is 93% identical to the nucleotide sequence of the mouse SMAD6 gene (Imamura et al., 1997, Nature 389: 622-626). The rchd534-long sequence was reported in Hata et al., 1998, Genes and Development 12: 186-197.



**EXHIBIT B**  
**MARKED VERSION OF THE AMENDED CLAIMS**  
**CONTINUATION APPLICATION OF U.S. APPLICATION NO. 09/288,292**  
**FILED ON FEBRUARY 8, 2002**

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02/08/02

1. (Once Amended) A homozygous transgenic [non-human] animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, and wherein said animal displays a cardiovascular disease symptom.

2. (Once Amended) The transgenic animal of claim 1, wherein said cardiovascular disease symptom is hyperplasia, thickening of at least one cardiac valve, cardiac outflow tract development defects, cardiovascular calcification, epicardial vascular malformations, endocardial vascular malformation, or defects in the regulation of vascular tone.

3. (Once Amended) The transgenic animal of claim 1, wherein said cardiovascular disease symptom is cardiovascular calcification.

4. (Once Amended) The transgenic animal of claim 1, wherein said cardiovascular disease symptom is aortic or valvular calcification.

6. (Once Amended) A cell having a mutated rchd534 gene isolated from the transgenic animal of claim 1, wherein said cell is isolated from tissue displaying a cardiovascular disease symptom.

10. (Once Amended) A method of producing a [non-human] homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, comprising introducing a polynucleotide into an embryonic cell of said animal through homologous recombination with an endogenous rchd534 gene, and wherein said transgenic animal displays a cardiovascular disease symptom.

26. (Once Amended) A method for identifying a substance for treating or preventing cardiovascular disease, comprising administering said substance to a [non-human] homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, wherein said transgenic animal displays a cardiovascular disease symptom, and wherein amelioration of said cardiovascular disease symptom indicates a substance effective in the treatment or prevention of cardiovascular disease.

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**EXHIBIT C**  
**PENDING CLAIMS OF**  
**CONTINUATION APPLICATION OF U.S. APPLICATION NO. 09/288,292**  
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1c971 U.S. PTO  
10/067741  
02/08/02

1. A homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, and wherein said animal displays a cardiovascular disease symptom.

2. The transgenic animal of claim 1, wherein said cardiovascular disease symptom is hyperplasia, thickening of at least one cardiac valve, cardiac outflow tract development defects, cardiovascular calcification, epicardial vascular malformations, endocardial vascular malformation, or defects in the regulation of vascular tone.

3. The transgenic animal of claim 1, wherein said cardiovascular disease symptom is cardiovascular calcification.

4. The transgenic animal of claim 1, wherein said cardiovascular disease symptom is aortic or valvular calcification.

5. The animal of claim 1 which is a mouse.

6. A cell having a mutated rchd534 gene isolated from the transgenic animal of claim 1, wherein said cell is isolated from tissue displaying a cardiovascular disease symptom.

7. The cell of claim 6, wherein said symptom is hyperplasia, thickening of at least one cardiac valve, cardiac outflow tract development defects, cardiovascular calcification, epicardial vascular malformation, endocardial vascular malformation, or defects in the regulation of vascular tone.

8. The cell of claim 6, wherein said symptom is cardiovascular calcification.

9. A cell line established from the cell of claim 6, wherein said cell is isolated from a tissue which exhibits at least one of the following cardiovascular developmental phenotypes: hyperplasia, thickening of at least one cardiac valve, cardiac outflow tract development defects, aortic ossification, epicardial vascular malformation, endocardial vascular malformation, or defects in the regulation of vascular tone.

10. A method of producing a homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, comprising introducing a polynucleotide into an embryonic cell of said animal through homologous recombination with an endogenous rchd534 gene, and wherein said transgenic animal displays a cardiovascular disease symptom.

11. The method of claim 10, wherein said cardiovascular disease symptom is hyperplasia, thickening of at least one cardiac valve, cardiac outflow tract development defects, cardiovascular calcification, epicardial vascular malformation, endocardial vascular malformation, or defects in the regulation of vascular tone.

12. The method of claim 10, wherein said cardiovascular disease symptom is cardiovascular calcification.

13. The animal of claim 10, wherein said animal is a mouse.

26. A method for identifying a substance for treating or preventing cardiovascular disease, comprising administering said substance to a homozygous transgenic animal having a mutated rchd534 gene, wherein the wild-type rchd534 gene has been replaced with a rchd534-LacZ gene which lacks the MH2 domain encoding region, wherein said transgenic animal displays a cardiovascular disease symptom, and wherein amelioration of said cardiovascular disease symptom indicates a substance effective in the treatment or prevention of cardiovascular disease.

